

Sex-dependent differences in phenobarbitone-induced oestradiol-2-hydroxylase activity in rat liver

C. N. THERON, A. C. NEETHLING, J. J. F. TALJAARD

Summary

Oestradiol-2-hydroxylase (E_2 -OH) activity was measured in liver and brain microsomes of 6-8-week-old Wistar rats. Phenobarbitone (75 mg/kg daily for 3 days) significantly increased enzyme activity in the liver of males and females, but there were striking differences between the two sexes. In males the enzyme activity was increased by 37% over control values and in females by 200%. The total microsomal cytochrome P-450 content was increased by 75% in males and by 82% in females. The apparent Michaelis constant (K_m) of E_2 -OH for 17 β -oestradiol in untreated males (9,8 μ M) and females (9,2 μ M) did not differ significantly. Phenobarbitone treatment, however, tended to reduce the apparent K_m in males (8,2 μ M) and to increase it in females (18,7 μ M). E_2 -OH activity was also detected in brain tissue of both sexes, but it was 50-200-fold lower than in the liver and was not increased by phenobarbitone.

S. Afr. med. J., 60, 279 (1981).

The existence of sexual differences in the hepatic metabolism of steroids and drugs is well known.^{1,2} Male rats, for example, metabolize hexobarbitone and ethylmorphine 2-4 times faster than females.³ These sex-dependent patterns of drug metabolism appear to be imprinted by the steroid environment to which the animal is exposed in the neonatal period. The male pattern of ethylmorphine metabolism in rats, for example, is dependent on the presence of testosterone shortly after birth.⁴ Over the past few years it has also become clear that the pituitary gland may be involved in maintaining the neonatally imprinted sexual patterns of hepatic drug metabolism.¹

Several steroid-metabolizing enzymes which show sex-dependent differences in activity have been studied, but relatively little is known about oestradiol-2-hydroxylase (E_2 -OH) in this respect. In recent years this enzyme has attracted considerable attention because it catalyses the formation of catechol oestrogens which are thought to play an important role in neuro-endocrine regulation.⁵⁻⁹ The recent introduction of an extremely sensitive assay for E_2 -OH⁷ has greatly simplified the investigation of this enzyme. It has been detected in several tissues, the liver having by far the highest activity, followed by

the brain.⁸ Most of the enzyme activity is present in microsomes and there is good evidence that E_2 -OH belongs to the cytochrome P-450 group of mono-oxygenases.⁷⁻⁸ The activity of the enzyme is higher in male rats than in females and it is lowered in males by castration.⁸

We undertook the present investigation to obtain more information about sex-dependent differences in E_2 -OH activity. In addition we wanted to find out whether it can be induced by phenobarbitone treatment, since this drug is known to increase the metabolic clearance rate of many steroids.¹⁰ Furthermore, phenobarbitone treatment of pregnant rats affects the sexual functions of female offspring.¹¹ In the present article we shall present evidence that phenobarbitone treatment increases the E_2 -OH activity in the liver of Wistar rats and that there are important sexual differences in this respect.

Materials and methods

Chemicals

All chemicals, unless otherwise specified, were of analytical grade and were obtained from E. Merck, Darmstadt, West Germany. The 17 β -oestradiol was purchased from Sigma Chemical Co., St Louis, Mo., USA, and NADPH from Boehringer Mannheim, Mannheim, W. Germany. Catechol-*o*-methyltransferase (COMT) was prepared by us from fresh ox liver, essentially according to the method described by Axelrod and Tomchick¹² and modified by Nikodejevick *et al.*¹³ S-[³H] methyl adenosylmethionine (specific activity 11,8-13,5 Ci/mmol) was obtained from New England Nuclear Corp., Cambridge, Mass., USA.

Animals and drug treatment

Male and female Wistar rats, 6-8 weeks old and weighing 150-200 g, were used. They were housed in plastic cages with wire mesh flooring under diurnal lighting and they had free access to food (standard Epol pellets) and water. The rats were injected intraperitoneally with sodium phenobarbitone dissolved in 0,9% NaCl or with 0,9 NaCl only (controls). The phenobarbitone injection schedule for each animal was 75 mg/kg once a day for 3 days. After the last injection the rats were fasted for 18 hours before being killed, but water was allowed freely.

Preparation of microsomes

The rats were killed by decapitation and tissues were rapidly removed and placed in ice-cold buffered isotonic KCl (0,154M KCl: 50 mM tris, pH 7,4). For each experiment tissues from 3-6 animals (control and phenobarbitone-treated groups respectively) were pooled. Livers were minced with scissors before homogenization. The tissues were homogenized in the buffered isotonic KCl (4 ml/g tissue) in a Potter-Elvehjem homogenizer with a Teflon pestle driven by an 1/15 hp stirrer (Tri-R instruments, Rockville Centre, NY, USA). For both brain and liver 10 passes at half the maximum setting of the instrument were used.

MRC Neurochemistry Group, Department of Chemical Pathology, University of Stellenbosch, Parowvallei, CP

C. N. THERON, M.SC. (MED. SCI.)

A. C. NEETHLING, M.SC., PH.D.

J. J. F. TALJAARD, M.B. CH.B., M.D.

Date received: 16 April 1981.

Microsomes were prepared by means of standard differential centrifugation methods. Brain homogenates were centrifuged once at 12 000 *g* for 20 minutes and the resulting supernatant was then centrifuged at 105 000 *g* for 60 minutes to pellet microsomes. The liver homogenates were treated slightly differently in that they were centrifuged twice at 12 000 *g* for 15 minutes. The second 12 000 *g* supernatant was then carefully aspirated off to about 2 cm above the pellet and centrifuged at 105 000 *g* for 60 minutes. Microsomal pellets were rinsed twice in ice-cold isotonic KCl and were then suspended by homogenization in isotonic KCl-tris to give protein concentrations of about 3 mg/ml (brain) and 10 mg/ml (liver). The protein concentration of microsomal suspensions was measured by the method of Lowry *et al.*¹⁴ Tissues and microsomes were kept at 0-4°C during the preparation.

Oestradiol-2-hydroxylase assay

E₂-OH activity was assayed by the method of Paul *et al.*⁷ In this method the hydroxylation of 17β-oestradiol is coupled with rapid *o*-methylation by means of COMT. A radioactive methyl group is derived from S-[³H]-methyl adenosylmethionine. The resulting ³H-methoxy oestrogens are stable and can be extracted with *n*-heptane. The specificity of the assay has been confirmed by chromatographic and mass spectroscopic analysis of the heptane-extractable radioactive products. More than 90% of these represented methoxy-oestrogens (2-methoxy-oestradiol and 2-hydroxy-oestradiol-3-methyl ether⁷).

Each reaction tube contained the following reagents: 5 μl 0,154M KCl, 50mM tris (pH 7,4), 10μl 1M MgCl₂, 5 μl (2,5 μCi) S-[³H]-methyl adenosylmethionine (sp. activity 11,8-13,5 Ci/mmol), 20 μl COMT, 5 μl 10 mM ascorbic acid, 5 μl 0,0135M NADPH, 50 μl microsomal suspension and 17 β-oestradiol in 2 μl absolute ethanol. All the reagents, except S-[³H]-methyl adenosylmethionine, COMT and oestradiol, were dissolved in the buffered isotonic KCl (pH 7,4) and were freshly prepared just before the experiment. Each reaction tube contained 20-30 μg microsomal protein (liver), or 100-150 μg microsomes (brain). The total volume of the reaction mixture was 102 μl and it was kept in crushed ice until all the reagents had been added. Blanks, consisting of all the reagents plus 50 μl of heat-inactivated microsomes, were included in all the experiments.

The reaction was started by placing the tubes in a shaking water-bath at 37°C. After 10 minutes' incubation the reaction was stopped by adding 0,5 ml 0,5M borate buffer (pH 10). The radioactive methoxy-oestrogen was then extracted with 6 ml heptane by vigorous shaking for 30 seconds, followed by centrifugation to separate the heptane from the watery phase. Two-millilitre aliquots of the heptane extract were then pipetted into counting vials and evaporated to dryness in an oven at 90°C. To each vial 10 ml Insta-Gel (Packard Instrument Co., Downer's Grove, Ill., USA) was added and radioactivity

measured in a Beckman LS 9000 scintillation counter. The amount of methoxy-oestrogen formed was expressed as picomoles per milligram microsomal protein per 10 minutes' incubation and was directly calculated from the specific activity of the S-[³H]-methyl adenosylmethionine.

Apparent Michaelis constant (K_m)

The apparent K_m of E₂-OH was determined by assaying as described above with different concentrations of 17β-oestradiol ranging from 7 to 150 μM. The reciprocals of the substrate concentrations were then plotted against the reciprocals of the reaction velocities (Lineweaver-Burke plot) to determine the relevant kinetic parameters.

Cytochrome P-450 measurement

The total cytochrome P-450 content of liver microsomes was measured essentially as described by Estabrook *et al.*¹⁵ Microsomes were suspended in 0,154M KCl, 50 mM tris (pH 7,4) to give a protein concentration of 1-2 mg/ml. Six millilitres of the suspension was then gassed with carbon monoxide for 2-3 minutes and the suspension equally divided into two cuvettes and a baseline of equal light absorbance recorded with a Beckman 5260 dual-beam spectrophotometer. A few crystals of sodium dithionite were then added to the sample cuvette and the difference spectrum was recorded between 390 and 510 nm. The CO complex of reduced cytochrome P-450 gave a well-defined absorbance peak at 450 nm. By using a millimolar extinction coefficient of 100 for the difference in absorbance at 450 minus 510, cytochrome P-450 could be quantified.

Results

Oestrogen-2-hydroxylase activity in brain and liver tissue

The E₂-OH activities in brain and liver are compared in Table I. In both sexes the liver had a much higher enzyme activity than the brain. Furthermore, enzyme activity in the liver of males was about fourfold higher than in females. There was, however, no significant difference between the sexes in respect of enzyme activity in the brain.

Effect of phenobarbitone treatment on enzyme activity

Phenobarbitone, in a daily dose of 75 mg/kg for 3 days, had no significant effect on E₂-OH activity in the brain tissue of male or female rats. In the liver, on the other hand, phenobarbitone caused a significant increase in enzyme activity in both sexes (Table I). There was, however, a most striking difference

TABLE I. E₂-OH ACTIVITY* IN BRAIN AND LIVER OF RATS

Treatment	Brain				Liver			
	Male		Female		Male		Female	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
None	4,9	±0,712	6,0	—	1 477	± 68,41	367	± 47,62
		(3)	(1)			(7)		(2)
Phenobarbitone	4,2	±0,498	5,4	—	2 029	±119,22	1 108	±116,02
		(3)	(1)			(6)		(2)
	NS		NS		P < 0,01		P < 0,01	

* E₂-OH activity is expressed as pmol methoxy-oestrogen formed per mg microsomal protein per 10 minutes' incubation at 37°C. The values given represent the means (± SE) of the number of experiments (N) stated in brackets. In each experiment tissues from 3-6 animals were pooled. Statistical significance was determined by means of the *t* test.

between the sexes in the magnitude of the enzyme-inducing effect. The increased enzyme activity in females (200%) was about fivefold greater than the increase observed in males (37%) (Table II).

TABLE II. COMPARISON OF E₂-OH AND TOTAL CYTOCHROME P-450 INDUCTION IN LIVERS OF MALE AND FEMALE RATS

Treatment	Male		Female	
	E ₂ -OH	Cyt. P-450	E ₂ -OH	Cyt. P-450
None	1 477	0,60	367	0,45
Phenobarbitone	2 029	1,05	1 108	0,82
% induction	+37%	+75%	+200%	+82%

E₂-OH activity is expressed as pmol methoxy-oestrogen per mg microsomal protein formed per 10 min at 37°C. Cytochrome P-450 concentration is given in nmol per mg microsomal protein.

Effect of phenobarbitone treatment on total cytochrome P-450 in liver microsomes

There is evidence that E₂-OH belongs to the microsomal cytochrome P-450 group of mono-oxygenases.^{7,8} In view of this we were interested in finding out whether induction of cytochrome P-450 in liver microsomes could be correlated with the induction of E₂-OH activity. The values for cytochrome P-450 in liver microsomes of control and phenobarbitone-treated rats are given in Table II. As can be seen, microsomes from male rats contained slightly more cytochrome P-450 than those from females. In both sexes phenobarbitone caused a substantial increase in the amount of cytochrome P-450, but there was no significant difference between males and females in the magnitude of induction when compared with the respective control values.

Effect of phenobarbitone treatment on enzyme kinetics

In an attempt to gain more insight into the sexual differences in E₂-OH induction we measured the apparent K_m of the enzyme in control and phenobarbitone-treated rats. The results of these experiments are shown in Figs 1 and 2 and in Table III. In males phenobarbitone tended to decrease the apparent K_m (Fig. 1). In females, on the other hand, we observed an increase in the K_m values over those of control animals (Fig. 2).

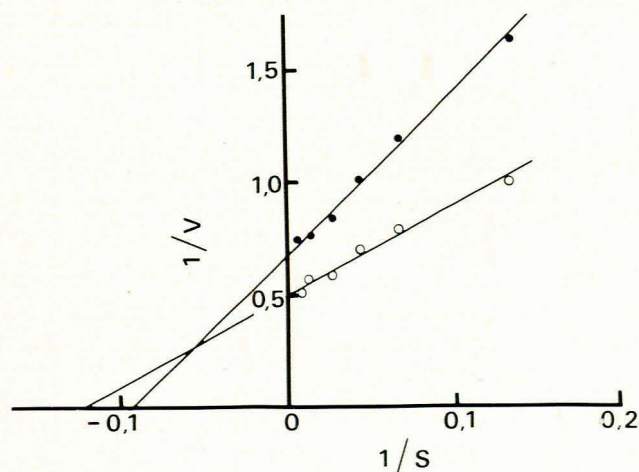


Fig. 1. Effects of phenobarbitone treatment on apparent K_m of E₂-OH in liver microsomes of male rats. The plot represents one experiment. Closed circles: control (K_m = 11,4 μM); open circles: phenobarbitone-treated animals (K_m = 8,2 μM). Values for 1/v were multiplied by 10³.

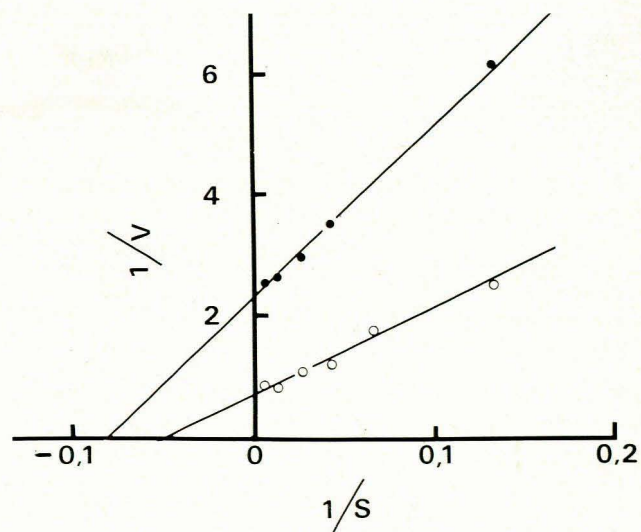


Fig. 2. Effect of phenobarbitone treatment on apparent K_m of E₂-OH in liver microsomes of female rats. The plot represents one experiment. Closed circles: control (K_m = 12,5 μM); open circles: phenobarbitone-treated animals (K_m = 21,5 μM). Values for 1/v were multiplied by 10³.

TABLE III. EFFECT OF PHENOBARBITONE ON THE APPARENT K_m OF E₂-OH IN LIVER

Treatment	Male		Female	
	Mean	SEM	Mean	SEM
None	9,8	±0,564 (4)	9,2	±3,300 (2)
Phenobarbitone	8,15	±0,050 (2)	18,7	±2,85 (2)

K_m values are given in μM and were obtained from Lineweaver-Burke plots as shown in Figs. 1 and 2. The values represent the means obtained from the number of experiments shown in brackets.

Discussion

In the present study we have confirmed earlier observations^{7,8} which indicate that rat brain tissue contains E₂-OH. Although the function of this enzyme in the brain is not known, there is growing evidence that it may be involved in neuro-endocrine regulation through its ability to form catechol oestrogens. Some of this evidence has been summarized in a recent review⁹ and research papers.^{16,17} The catechol oestrogens may conceivably affect neuro-endocrine function by virtue of their oestrogenic or anti-oestrogenic properties.⁹ Another possibility is that they could affect the hypothalamic-pituitary axis by inhibiting catecholamine synthesis. Evidence for this possibility is the observation that catechol oestrogens inhibit tyrosine hydroxylase activity in hypothalamic extracts.¹⁸

Since there is evidence that E₂-OH belongs to the cytochrome P-450 group of mono-oxygenases, the question arises whether its activity in the brain is inducible by drugs. In the present study phenobarbitone had no statistically significant effect on E₂-OH activity in the brain. Hoffman *et al.*⁸ also found that cytochrome P-450 inducers did not increase the activity of this enzyme in the brain. They did, however, find evidence for hormonal effects on its activity. Thus castration caused a fall in enzyme activity in both liver and brain tissue of male rats. Hoffman *et al.*⁸ also observed a higher E₂-OH activity in the brains of males than in those of females, but we were unable, in the present study, to confirm these sex differences. Although the reason for these contradictory results is not clear to us, there is at least one important factor which could influence comparisons between male and female rats, and that is the oestrus cycle. Fishman *et al.*⁵

have reported significant changes in E_2 -OH activity in the brains of female rats during the oestrus cycle. During oestrus, for example, enzyme activity was nearly 6 times higher than during di-oestrus.

The stimulatory effect of phenobarbitone on E_2 -OH activity in the liver is in sharp contrast to its inability to induce this enzyme in the brain. Percy and Shanley¹⁹ have also found that phenobarbitone does not induce cytochrome P-450 in the microsomes or mitochondria of rat brain. Furthermore, Liem *et al.*²⁰ have reported tissue specificity with respect to the induction of certain forms of cytochrome P-450.

To our knowledge the induction of hepatic E_2 -OH activity by phenobarbitone has not been reported before. In fact, other workers have been unable to induce this steroid-metabolizing enzyme in the liver of male rats with phenobarbitone or 3-methylcholanthrene.⁸⁻²¹ On the other hand, the induction of several other steroid-metabolizing enzymes has been described.^{10,22,23} The fact that phenobarbitone and other clinically used drugs may alter the metabolism of steroids is of practical concern to physicians and the importance of this is attested to by a recent review.¹⁰

A very interesting observation in our present study was that phenobarbitone affected the kinetic characteristics of E_2 -OH differently in male and female rats. It caused a significant increase in enzyme activity (V_{max}) in both sexes, but with the increased activity in females about fivefold greater than in males. Secondly, phenobarbitone treatment tended to decrease the apparent K_m of the enzyme in males and to increase it in females. The changes in the mean K_m values for each sex did not reach statistical significance with the small number of experiments reported here. Nevertheless the relatively large differences seen in individual experiments with female rats were highly suggestive of a real effect of phenobarbitone on the K_m .

The possibility that phenobarbitone has different effects on the kinetic characteristics of E_2 -OH in males and females is interesting, particularly in view of the fact that the V_{max} and K_m of other steroid-metabolizing enzymes show sex-dependent differences.¹ Also important in this context is the finding by Chung² that the K_m and V_{max} of ethylmorphine *N*-demethylase in liver microsomes are both imprinted by androgens in the neonatal male rat, but that the sensitive periods for imprinting of the two kinetic characteristics differ. Furthermore, the maintenance of the imprinted K_m and V_{max} requires the presence of an intact pituitary gland.

In female rats an increased K_m and V_{max} associated with phenobarbitone treatment may reflect the induction of a form of cytochrome P-450 which hydroxylates oestradiol, but with a lower affinity towards this substrate. It is now widely accepted that there are multiple forms of cytochrome P-450, each with its own spectrum of activity towards endogenous substrates and foreign compounds.^{20,21} Furthermore, more than one form of cytochrome P-450 may be active towards the same substrate. Kremers *et al.*²⁴ studied steroid 16α -hydroxylase activities in normal and phenobarbitone-treated rats and used four different substrates to measure enzyme activity. They concluded that steroid 16α -hydroxylase activity may be due to at least two different forms of cytochrome P-450. Some of the observations which these authors made are remarkably similar to our findings. The activity of 16α -hydroxylase with pregnenolone as substrate, for example, was differently induced by phenobarbitone in males and females. In females the induced enzyme activity was roughly tenfold higher than control values, whereas the induced activity in males was only about twofold higher than control values. Kremers *et al.*²⁴ also found, as we did, that the variations in

inducibility and in male-female enzyme activity ratios could not be directly correlated with changes in the total cytochrome P-450 content of microsomes.

While we need more evidence on this point, it is tempting to explain our findings in terms of factors which control neonatally imprinted sexual differences in the adult animal. Sex-dependent differences in the oxidative metabolism of several endogenous steroids have been reported.^{1,2} Although relatively little is known about this form of sexual dimorphism, some of the major factors which appear to control it have been identified. The basic masculine or feminine patterns of steroid metabolism are established soon after birth by the gonadal steroids to which the young animal is exposed. Thus the male pattern in rats depends on the presence of testosterone, whereas the female pattern develops in the absence of testosterone.⁴ In addition to neonatal imprinting there are also two known factors which are necessary to maintain sexual dimorphism in the adult animal. These factors are the pituitary gland and the circulating sex steroids. Castration of male rats, or testosterone treatment of females, for example, results in decreases or increases of hepatic steroid and drug metabolism.² Hypophysectomy of female rats leads to a drug-metabolizing pattern in the liver which resembles that of the male.²⁵

In conclusion, and as a final summary of our present findings, we would like to propose the following working hypothesis: sex-dependent differences in E_2 -OH activity in rat liver are imprinted neonatally and the male and female patterns of enzyme activity are maintained by the pituitary gland. Phenobarbitone treatment may affect E_2 -OH activity in the liver by a dual mechanism, i.e. through suppression of the hypothalamic-pituitary axis and by direct induction of the enzyme. Whether these effects of phenobarbitone are implicated in long-term changes in female reproductive function of rats exposed to phenobarbitone *in utero*¹¹ remains to be established.

REFERENCES

- Gustafsson, J. A. and Skett, P. (1979): *Acta biol. med. germ.*, **38**, 307.
- Chung, L. W. K. (1977): *Biochem. Pharmacol.*, **26**, 1979.
- Schenkman, J. B., Frey, I., Remmer, H. *et al.* (1967): *Molec. Pharmacol.*, **3**, 516.
- Chung, L. W. K., Raymond, G. and Fox, S. (1975): *J. Pharmacol. exp. Ther.*, **193**, 621.
- Fishman, J., Norton, B. I. and Krey, L. (1980): *Biochem. biophys. Res. Commun.*, **93**, 471.
- Lloyd, T. and Ebersole, B. J. (1980): *J. Neurochem.*, **34**, 726.
- Paul, S. M., Axelrod, J. and Diliberto, E. J. (1977): *Endocrinology*, **101**, 1604.
- Hoffman, A. R., Paul, S. M. and Axelrod, J. (1980): *Biochem. Pharmacol.*, **29**, 83.
- Neethling, A. C. and Taljaard, J. J. F. (1980): *S. Afr. med. J.*, **58**, 495.
- Elias, A. N. and Gwinup, G. (1980): *Metabolism*, **29**, 582.
- Gupta, C., Sonawane, B. R., Yaffe, S. J. *et al.* (1980): *Science*, **208**, 508.
- Axelrod, J. and Tomchick, R. (1958): *J. biol. Chem.*, **233**, 702.
- Nikodejevick, B. S., Senoh, S., Daly, J. W. *et al.* (1970): *J. Pharmacol. exp. Ther.*, **174**, 83.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. *et al.* (1951): *J. biol. Chem.*, **193**, 265.
- Estabrook, R. W., Petersen, J., Baron, J. *et al.* in Chignell, C. F., ed. (1972): *Methods in Pharmacology*, vol. 2, p. 303. New York: Appleton-Century Crofts.
- Adashi, E. Y., Rakoff, J., Divers, W. *et al.* (1979): *Life Sci.*, **25**, 2051.
- Fishman, J., Norton, B. I. and Hahn, E. F. (1980): *Proc. nat. Acad. Sci. (Wash.)*, **77**, 2574.
- Foreman, M. M. and Porter, J. C. (1980): *J. Neurochem.*, **34**, 1175.
- Percy, V. A. and Shanley, B. C. (1979): *Ibid.*, **33**, 1267.
- Liem, H. H., Muller-Eberhard, U. and Johnson, E. F. (1980): *Molec. Pharmacol.*, **18**, 565.
- Numazawa, M., Soeda, N., Kiyome, Y. *et al.* (1979): *J. Steroid Biochem.*, **10**, 227.
- Levin, W., Welch, R. M. and Conney, A. H. (1967): *Endocrinology*, **80**, 135.
- Nakamura, Y. and Ueda, S. (1980): *Biochem. biophys. Res. Commun.*, **93**, 1014.
- Kremers, P., Pasleau, F. and Gielen, J. E. (1978): *Ibid.*, **84**, 706.
- Gustafsson, J. A. and Stenberg, S. (1974): *Endocrinology*, **95**, 891.